Process for production of cephalosporin C

Field of the invention

The present invention relates to isolated nucleic acid molecules which code for a new protein from Acremonium chrysogenum, to vectors which comprise such a nucleic acid molecule, to Acremonium chrysogenum host cells which have been transformed with such a vector and to a method for production of cephalosporin C using such transformed host cells.

Background of the invention

Cephalosporin C is a natural metabolite which is obtained by fermentation of the filamentous fungus Acremonium chrysogenum (referred to as A. chrysogenum hereinbelow) on an industrial scale. This substance is an important precursor for a number of semi-synthetic cephalosporin antibiotics. The cephalosporin substance class is of great therapeutic importance. Increases in the yields of industrial cephalosporin fermentation depend essentially on continuous genetic strain improvement, in addition to improvements in process technology. More and more to the fore of modern methods of the said strain improvement is the transformation of producer strains with specific genes which have a potential for increasing production. A small group of known cephalosporin biosynthesis genes may be suspected, via the knowledge of biochemical relationships of cephalosporin biosynthesis, of having a strain improvement potential. Amplification, i.e. increasing the copy number of such known genes, indeed shows experimentally partly a significant improvement in the productivity of a producer organism. However, the group of known genes for which a strain improvement potential can be predicted from scientific plausibility assessments is very small. In addition to these known biosynthesis genes, however, an unknown number of further genes may be suspected which likewise cause a production-increasing potential by way of amplification. Frequently, the function of such genes is unknown, since there is currently still very little understanding of the entirety of cellular processes which influence cephalosporin biosynthesis. Strategies for identifying further genes with production-increasing potential are therefore of great importance.

It is thus a central object of the present invention to find such a further, hitherto unknown gene. It is thus an object of the present invention to provide a nucleic acid and vectors which

code for new protein from A. chrysogenum and can be used for transformation of an A. chrysogenum host cell so that this host cell is capable of providing cephalosporin C in good yields. It is another object of the present invention to provide such a transformed host cell. Finally, it is another object of the present invention to provide a process for production of cephalosporin C using the said transformed host cell.

Figures

Figure 1 shows the amino acid sequence (SEQ ID NO 1 = sequence identity No. 1) of a novel A. chrysogenum protein, which is deduced from a nucleic acid molecule of the invention (nucleic acid sequence according to Figure 2 or 4). The sequence is shown from the N terminus to the C terminus.

Figure 2 (SEQ ID NO 2) shows the genomic DNA sequence, including the 3 introns of the coding region of a genomic clone of the A. chrysogenum gene found within the framework of the present invention from the translation start codon (ATG) to the last coding codon (TGG). The introns are underlined. The sequence is depicted in the form of a single strand in 5'-to-3' orientation.

Figure 3 (SEQ ID NO 3) shows the cDNA sequence of the coding region of the new gene from the translation start codon (ATG) to the last coding codon (TTG); the sequence is depicted in the form of a single strand in 5'-to-3' orientation.

Figure 4 (SEQ ID NO 4) shows the genomic DNA sequence of a BamHI/EcoRI fragment of a genomic clone of the new gene (the sequence is depicted in the form of a single strand in 5'-to-3' orientation). The translation start codon (ATG) and the translation stop codon (TAA) of the coding region are depicted underlined and in bold type. The introns are depicted underlined.

Figure 5 (SEQ ID NO 5) shows the A. chrysogenum genomic DNA sequence of an approx. 16 kb region marked by cleavage sites for SnaB1 and Bfr1, which comprises the biosynthesis genes pcbC (position 1366 to position 350, inverse arrangement) and pcbAB (position 2598 to position 13517). The sequence is depicted in the form of a single strand in 5'-to-3' orientation. The particular translation start codons and the particular translation stop

codons of the corresponding coding regions are depicted underlined and in bold type. The cleavage sites mentioned are depicted underlined.

Figure 6 (SEQ ID NO 6) shows the A. chrysogenum genomic DNA sequence of an approx. 5.8 kb region marked by cleavage sites for EcoRV and BamHI, which comprises the biosynthesis genes cefD1 (position 2372 to position 180, inverse arrangement) and cefD2 (position 3888 to position 5133). The sequence is depicted in the form of a single strand in 5'-to-3' orientation. The particular translation start codons and the particular translation stop codons of the corresponding coding regions are depicted underlined and in bold type. The intron in cefD2 and the cleavage sites mentioned are depicted underlined.

Figure 7 (SEQ ID NO 7) shows the A. chrysogenum genomic DNA sequence of an approx. 4.6 kb region marked by cleavage sites for Xba1 and SgrAI, which comprises the biosynthesis genes cefEF (position 1118 to position 122, inverse arrangement) and cefG (position 2058 to position 3534). The sequence is depicted in the form of a single strand in 5'-to-3' orientation. The particular translation start codons and the particular translation stop codons of the corresponding coding region are depicted underlined and in bold type. The two introns in cefG and the cleavage sites mentioned are depicted underlined.

Detailed description of the invention

A new gene in A. chrysogenum, which codes for a hitherto unknown protein in A. chrysogenum, was found within the framework of the present invention. The new gene is in its native state located approx. 5.5 kb (kilo bases) downstream (read in the direction of translation) of the known A. chrysogenum cefEF gene (S.E. Samson et al., Biotechnology 5 (1987), 1207-1214) on the same chromosome in the A. chrysogenum genome.

The new gene was found in the A. chrysogenum strain ATCC48272 (obtainable with this number from the ATCC, American Type Culture Collection, PO Box 1549, Manassas, VA 20108, USA).

This strain has already been characterized in detail, for example as a producer of cephalosporins, in particular cephalosporin C (L.H. Malmberg and W.S. Hu, Appl. Microbiol. Biotechnol. 38 (1992), 122-128; Y.Q. Shen et al., Bio-Technology 4 (1986), 61-64);

isopenicillin N synthetase (I.J. Hollander et al., Science 224 (1984), 610-612; J.M. Luengo et al., Bio-Technology 4 (1986), 44-47); deacetoxy-cephalosporin C synthetase (Y.Q. Shen et al., Enzyme Microb. Technol. 6 (1984), 402-404); and ACV synthetase (J. Zhang et al., Curr. Microbiol. 18 (1989), 361-367; J. Zhang and A.L. Demain, Arch. Microbiol. 158 (1992), 364-369).

However, the new gene can also be found in other A. chrysogenum strains and be isolated therefrom. Alternatively, the nucleic acid and amino acid sequences or molecules presented herein may be synthesized, in particular chemically synthesized.

The gene codes for a protein of 526 amino acids in length (see Figure 1, SEQ ID NO 1). The amino acid sequence is depicted in Figure 1. The coding region in the gene is interrupted by 3 introns, as Figures 2 and 4 show.

The present invention thus relates to an isolated nucleic acid molecule which codes for a protein comprising the amino acid sequence according to SEQ ID NO 1 (see Figure 1).

A nucleic acid molecule of this type may thus code, for example, for a protein which comprises, in addition to the amino acid sequence listed (SEQ ID NO 1), still further amino acids, for example for a fusion protein. Such fusion proteins may play a part, for example, if preparation of the new protein in isolated form is desired. The fusion parts can increase stability or facilitate purification, for example.

Within the framework of the present invention, preference is given to a nucleic acid molecule of the invention, which codes only or exclusively for an amino acid sequence according to SEQ ID NO 1. A nucleic acid molecule of this type may advantageously be employed for the purpose of producing cephalosporin C, described hereinbelow. The present invention thus further relates to a nucleic acid molecule of the invention, which codes for a protein consisting of the amino acid sequence according to SEQ ID NO 1.

A nucleic acid molecule of the invention is preferably a DNA molecule, in particular an isolated genomic DNA molecule or a corresponding cDNA molecule. A cDNA molecule may be prepared, for example, by reverse transcription of a corresponding mRNA molecule or

synthetically. Alternatively, the nucleic acid molecule may be an RNA molecule, in particular an mRNA molecule.

The DNA molecule of the invention may be prepared, for example, by generating a genomic DNA library of the genome of the said A. chrysogenum strain ATCC48272. A genomic clone is identified which contains the known A. chrysogenum cefEF gene and additionally at least about 10 kb of sequence downstream of the cefEF gene. This may be carried out by screening with homologous probes whose structures can be deduced from the known nucleic acid sequence of the cefEF gene (S.E. Samson et al., see above). Appropriate techniques are known from the literature (e.g. in T. Maniatis et al., Molecular Cloning - A Laboratory Manual, 1982, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA). The desired DNA molecule is located on an approx. 2.5 kb EcoRI/BamHI fragment of such a clone, which fragment can be isolated or prepared using classical techniques. Fig. 4 depicts such a fragment. A preferred embodiment of the present invention thus relates to a nucleic acid molecule of the invention, comprising a base sequence according to SEQ ID NO 4 or a base sequence which differs from the sequence according to SEQ ID NO 4 only due to the degeneracy of the genetic code. That is to say, according to the invention the present invention also relates to those nucleic acid molecules which differ from the specifically listed sequences by one or more of the codons listed being replaced by one or more other codons in such a way that the amino acid sequence of the encoded protein (SEQ ID NO 1) is not altered. This also applies to the further nucleic acid molecules described below. The nucleic acid molecule according to SEQ ID NO 4 contains regulatory sequences (such as a promoter and a stop codon) and may be used advantageously, in particular in a vector, for transformation of A. chrysogenum and thus for production of cephalosporin C.

The mentioned EcoRI/BamHI fragment of 2.5 kb comprises in particular the coding part of the new gene. This part is depicted in Fig. 2 and comprises 3 introns. The present invention thus further relates to a nucleic acid molecule of the invention, comprising a base sequence according to SEQ ID NO 2 or a base sequence which differs from the sequence according to SEQ ID NO 2 only due to the degeneracy of the genetic code, as discussed above. A nucleic acid molecule of this type thus corresponds to the genomic DNA sequence of the coding part of the new gene. Further preferred embodiments of the present invention are those nucleic

acid molecules which differ from that of SEQ ID NO 2 by the absence of one, two or all three introns.

Therefore, preference is furthermore given to a nucleic acid molecule of the invention, comprising a base sequence according to SEQ ID NO 3 or a base sequence which differs from the sequence according to SEQ ID NO 3 due to the degeneracy of the genetic code, as discussed above. A nucleic acid molecule of this type no longer comprises any of the introns mentioned and as such can be equated with a corresponding cDNA sequence.

A nucleic acid molecule of the invention (including a cDNA molecule mentioned) may furthermore, for example, be fully or partly synthesized. RNA or mRNA molecules of the invention can be isolated from the microorganism A. chrysogenum by means of standard techniques or can be produced synthetically. It is possible to prepare from an appropriate mRNA a corresponding cDNA molecule, using standard techniques.

While it is perfectly possible for the said nucleic acid molecules to contain further base sequences (in order to code for a fusion protein, for example), preferred embodiments relate to a nucleic acid molecule of the invention which exclusively or solely consists of a base sequence selected from the group of the base sequences SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4 and a base sequence which differs from any of the said sequences only due to the degeneracy of the genetic code, as discussed above.

In another embodiment nucleic acid molecules of the invention additionally comprise, immediately after the end of the coding region (corresponding to the C terminus of the encoded protein), one or more stop codon(s). Preference is given to the naturally occurring stop codon which has been identified as TAA. The other stop codons may also be used, however.

Owing to similarities with other, known protein sequences, the protein (SEQ ID NO 1) encoded by the nucleic acid molecules of the invention may be characterized as a hydrolase, in particular as a acetyl-CoA hydrolase, although this should not be construed as being limiting. The principal concern of the present invention, however, is not the description of the function of the new protein but, in particular, the use of the new gene or the use of the nucleic acid molecules of the invention which code for the new protein in order to transform,

preferably, A. chrysogenum host strains, in particular for the purpose of producing cephalosporin C, this being illustrated in more detail below.

The present invention furthermore relates to a vector comprising any of the mentioned nucleic acid molecules of the invention. Preferably, such a vector is suitable for transformation of a host cell. Such a host cell is in particular a microorganism, in particular A. chrysogenum. Such a vector may be in the form of a plasmid, for example, and contains, where necessary, in addition to a nucleic acid molecule of the invention further sequences, for example an origin of replication and further regulatory elements (promoter, transcription termination signal, translation start signal, translation termination signal, etc.) so that, after transformation has been carried out, expression of the nucleic acid molecule of the invention can take place. After transformation has been carried out, a nucleic acid molecule of the invention and also other vector elements may integrate into the genome of the host cell, amounting to an amplification of the coding part of the new gene. A vector of the invention advantageously comprises a nucleic acid molecule comprising a base sequence according to SEQ ID NO 4. Such a base sequence corresponds to the EcoRI/BamHI fragment mentioned and already comprises regulatory sequences such as, for example, a corresponding promoter.

Vectors of this type may be generated according to standard techniques by cloning a nucleic acid molecule of the invention into suitable standard vectors.

In addition to the nucleic acid molecules described which derive from the newly found gene, a vector of the invention may, in particular, contain one or more further nucleic acid molecule(s) derived from genes which are already known and which are involved in cephalosporin biosynthesis in A. chrysogenum, with particular mention being made of pcbAB (S. Gutierrez, J. Bacteriol. 173 (1991), pp. 2354-2365), pcbC (S. Gutierrez, J. Bacteriol. 173 (1991), pp. 2354-2365), cef D1 (R.V. Ullan et al., J. Biol. Chem. 277 (2002) pp. 46216 – 46225), cefD2 (R.V. Ullan et al., J. Biol. Chem. 277 (2002) pp. 46216 – 46225), cefEF (S. Gutierrez, J. Bacteriol. 174 (1992), pp. 3056 -3064) and cefG (S. Gutierrez, J. Bacteriol. 174 (1992), pp. 3056 -3064).

Thus, in another preferred embodiment, a vector of the invention, as described above, additionally comprises at least one further nucleic acid molecule coding for a protein selected

from the group of proteins encoded by the following Acremonium chrysogenum genes: pcbAB, pcbC, cefD1, cefD2, cefEF and cefG.

Preferably, a vector of the invention additionally comprises two further nucleic acid molecules coding for the proteins correspondingly encoded by the Acremonium chrysogenum genes pcbAB and pcbC, respectively.

Furthermore, a vector of the invention preferably additionally comprises two further nucleic acid molecules coding for the proteins correspondingly encoded by the Acremonium chrysogenum genes cefD1 and cefD2, respectively.

Furthermore, a vector of the invention preferably additionally comprises two further nucleic acid molecules coding for the proteins correspondingly encoded by the Acremonium chrysogenum genes cefEFand cefG, respectively.

The pcbAB sequence to be used preferably corresponds to the nucleotide sequence to be found in Figure 5. The pcbC sequence to be used preferably corresponds to the nucleotide sequence which is likewise to be found in Figure 5. The cefD1 sequence to be used preferably corresponds to the nucleotide sequence to be found in Figure 6. The cefD2 sequence to be used preferably corresponds to the nucleotide sequence which is likewise to be found in Figure 6. The cefEF sequence to be used preferably corresponds to the nucleotide sequence to be found in Figure 7. The cefG sequence to be used preferably corresponds to the nucleotide sequence which is likewise to be found in Figure 7.

The present invention further relates to a host cell which has been transformed with a vector of the invention, which vector comprises the new nucleic acid molecule of the invention and additionally, where appropriate, further nucleic acid molecules, as described above. The host cell is preferably a microorganism, in particular A. chrysogenum.

A host cell of this type, in particular one of A. chrysogenum, is transformed with a vector of the invention according to standard methods. Such a method is described, for example, in C. Nowak and U. Kück, Curr. Genet. 25 (1994), pp. 34-40.

Advantageously, a transformed A. chrysogenum host cell of the invention may be used for production of cephalosporin C. The present invention thus also relates to a process for production of cephalosporin C, comprising culturing of an A. chrysogenum host cell of the invention under conditions suitable for effecting production of cephalosporin C by the host cell.

Suitable culturing/fermentation techniques, for example in particular for A. chrysogenum, are known to the skilled worker in the field of antibiotics and have been employed in production of cephalosporin C for a long time.

In a preferred embodiment the process of the invention furthermore comprises isolation of the cephalosporin C produced. Cephalosporin C produced by a transformed A. chrysogenum host cell of the invention is usually secreted by the microorganism and can be purified or isolated from the culture supernatant by known techniques, for example chromatographic techniques.

Cephalosporin C generated according to the invention may preferably be reacted to give further derivatives with antibiotic properties.

An alternative application of the present invention relates to an isolated protein comprising an amino acid sequence according to SEQ ID NO 1. Such a protein also comprises, as mentioned, corresponding fusion proteins from which, where desired, a mature protein with an amino acid sequence according to SEQ ID NO 1 can be generated by cleaving. Preference is given to a protein of the invention, which exclusively or solely consists of the amino acid sequence according to SEQ ID NO 1.

A protein of the invention may be produced by culturing a suitable prokaryotic or eukaryotic host cell containing a suitable expression vector of the invention, which vector comprises a nucleic acid molecule coding for the said protein, under conditions leading to expression of the said protein. The protein may be purified and isolated using common techniques. Examples of suitable prokaryotic host cells in which, in particular, a cDNA of the invention is used are bacterial cells, e.g. E. coli; examples of suitable eukaryotic host cells are mammalian cells such as, for example, CHO or BHK cells.

Such proteins of the invention may be used in isolated form, for example in the synthetic or semi-synthetic production of cephalosporin C or derivatives thereof. An example of common practice would be the immobilization on a synthesis column on which a reaction takes place.

The references mentioned herein are herewith in their entirety incorporated by reference.

The present invention is illustrated in more detail by the examples below but is not limited thereto. The examples relate in particular to preferred embodiments of the present invention.

Examples

The materials and reagents mentioned herein are familiar to the skilled worker, commercially available or readily accessible and can be used according to the manufacturer's instructions.

Example 1: Identification of a new gene and a new protein from A. chrysogenum

DNA sequences for the cephalosporin biosynthesis genes and flanking sequences may be constructed with the aid of lambda clones. Such lambda clones can be isolated from a lambda gene bank containing DNA inserts of A. chrysogenum ATCC48272. The construction of lambda gene banks is mentioned, for example, in T. Maniatis et al. (see above), as is the screening of lambda gene banks by means of lambda-plaque hybridization. The DNA sequence information on cephalosporin biosynthesis genes from A. chrysogenum, required for this screening, can be made available by means of database searches (e.g. GENBANK). Examples of search terms suitable for this database request are the names for an appropriate biosynthesis gene (cefEF) which is to be present on the clone searched for. Cloning of the gene found within the framework of the present invention may start out from a lambda clone which is identified by means of plaque hybridization using sequence information of the cefEF gene, provided that this clone contains a DNA insert of at least about 10 kb of DNA sequence downstream of the stop codon of the cefEF gene. A BamHI/EcoRI restriction fragment of about 2.5 kb which fully includes the gene of the invention and thus, in particular, the nucleic acid molecules of the invention according to SEQ ID NO 2 and SEQ ID NO 4 is essential for subsequent cloning. This fragment can be identified, for example, by PCR using the primers PCR1f and PCR1r which are defined by the following sequences:

Primer PCR1f 5'- TTT GGG CGA GTG GGC TAA TA (SEQ ID NO 8)

Primer PCR1r 5'- CAA CAA CGC CTC TCC CGT CT (SEQ ID NO 9)

In order to clone the newly found gene or the nucleic acid molecules of the invention, the BamHI/EcoRI restriction fragment of about 2.5 kb is cloned into the vector pCN3 (described in Nowak and Kück, see above). This vector is distinguished by a modified tubulin resistance gene (Nowak and Kück, see above) which allows selection of Acremonium transformants. The unique EcoRI cleavage site on pCN3 is utilized for cloning. A ligation is carried out in three steps:

1) ligating the two DNA molecules in each case via a compatible EcoRI cleavage site
2) adapting the protruding ends with Klenow enzyme
3) linking the modified ends by further ligation

The ligation product is transformed into E. coli (e.g. DH5alpha strain) and produced there in an amount sufficient for the further transformation steps and purified. Due to the way of construction, it is also possible to obtain plasmids which contain the nucleic acid molecule in inverse orientation; in principle, however, these structures have the same functionality. E. coli clones containing the desired plasmid can be identified by PCR using the PCR primers mentioned, PCR1f and PCR1r; the resulting PCR product is 2.5 kb in length. These PCR analyses may be carried out using the Expand High Fidelity PCR system from Roche Applied Science, according to the specifications for the PCR reaction which are also supplied. Subsequent sequencing and evaluation results in the nucleic acid sequences depicted in Figures 2 (SEQ ID NO 2) and 4 (SEQ ID NO 4). A cDNA sequence according to Figure 3 (SEQ ID NO 3) and also the amino acid sequence of the encoded protein according to Figure 1 (SEQ ID NO 1) can then be deduced therefrom. In principle, it is possible to finally verify the cloning product by sequencing and sequence comparison with the DNA sequences depicted in Figures 2 and 4. A plasmid carrying the EcoRI/BamH fragment is referred to as plasmid 1 and used below.

Example 2: Transformation of A. chrysogenum

Plasmid 1 is transformed into A. chrysogenum ATCC48272 (see above) by means of a standard procedure for protoplast transformation. These methods are described, for example, in Nowak and Kück (see above) and involve selection of transformants on benomyl-containing nutrient agar. The properties of the modified tubulin gene CA_Tubulin(Tyr) on plasmid 1 and, respectively, pCN3, used for this selection, is described in Nowak and Kück (see above), as is the benomyl-containing nutrient agar required for selection. Transformants from this type of experiments can be assayed, for example by means of PCR, for the presence of the essential parts of plasmid 1. The PCR primers below allow such assaying:

Primer PCR2f 5'- GCA GAG CGC AGA TAC CAA (SEQ ID NO 10)

Primer PCR2r 5'- CGT GGA CTC CAA CGT CAA (SEQ ID NO 11)

The resulting PCR product is 8 279 bp in length. The PCR analyses may be carried out, for example, by means of the Expand Long Template PCR system from Roche Applied Science, according to the specifications for the PCR reaction which are also supplied.

Sensibly, a population of transformants with pCN3 is provided for control purposes in addition to a population of transformants with plasmid 1. These transformants can be analysed in PCR reaction mixtures using the following PCR primers:

Primer PCR3f 5'- TTC CAT CCA GCA CCT CAC (SEQ ID NO 12)

Primer PCR3r 5'- CTT AAT GCG CCG CTA CAG (SEQ ID NO 13)

The resulting PCR product is 2 290 bp in length.

Example 3: Production and isolation of cephalosporin C

The transformants generated in Example 2 are tested for cephalosporin production in flask fermentation experiments. Sensibly, in each case a population of about the same size of

approx. 500 transformants with plasmid 1 and of control transformants with pCN3 is compared in parallel. To this end, supernatants of these flask fermentations are evaluated by means of HPLC analysis. A corresponding method, including the accompanying analysis, is described, for example, in L. Karaffa et al., Appl. Microbiol. Biotechnol. 51 (1999), 633-638. In order to obtain statistically relevant amounts of data, these analyses are repeated several times (e.g. 6 times), with each repeat comprising in each case several (e.g. 4) parallel flask fermentations of each strain which are individually tested. It turns out that it is possible to identify in this way strains which originate from the transformation with plasmid 1 and which have reproducibly a distinctly higher cephalosporin productivity than the population of pCN3 control transformants in comparison therewith.

The HPLC columns used in the said analysis may also be used in the purification and isolation of cephalosporin C produced.

Example 4: Construction of plasmid 2 comprising the A. chrysogenum genes cefD1 and cefD2

Plasmid 1 of Example 1 is subsequently extended by the two cephalosporin biosynthesis genes cefD1 and cefD2. These genes are described in R.V. Ullan, J. Biol. Chem. 277 (2002), pp. 46216-46225. Both genes are located in close proximity on a 5.8 kb EcoRV/BamHI fragment and are provided with the aid of suitable lambda clones. Such lambda clones are isolated from a lambda gene bank containing DNA inserts of Acremonium chrysogenum ATCC48272. The construction of lambda gene banks is mentioned, for example, in T. Maniatis et al. (see above), as is the screening of lambda gene banks by means of lambda-plaque hybridization. The DNA sequence information of the biosynthesis genes cefD1 and cefD2, required for this screening, can be made available, for example, by means of database requests (e.g. GENBANK). Examples of search terms suitable for this database request are the names of the two biosynthesis genes (cefD1, cefD2). Cloning of the two biosynthesis genes starts out from a lambda clone which is identified by means of plaque hybridization using sequence information of the cefD1 and cefD2 genes, the said clone containing a DNA insert of at least 6 kb of DNA sequence. A BamHI/EcoRV restriction fragment of about 5.8 kb which comprises the two complete biosynthesis genes cefD1 and cefD2 is essential for subsequent cloning. This fragment can be identified by PCR using the primers PCR4f and PCR4r which are defined by the following sequences:

Primer PCR4f 5'- ATC TGA GTG GTT GTT CCG CG (SEQ ID NO 14)

Primer PCR4r 5'- CGA GGA TGA AGA CGG TGA AA (SEQ ID NO 15)

Plasmid 1 of Example 1 is extended by cloning the BamHI/EcoRV restriction fragment of about 5.8 kb according to SEQ ID NO 6, containing the two biosynthesis genes cefD1 and cefD2, into plasmid 1. The unique SmaI cleavage site on plasmid 1 is utilized for cloning. The two protruding ends of the 5.8 kb BamHI/EcoRV restriction fragment are adapted with Klenow enzyme and ligated with the SmaI-cleaved plasmid 1.

The ligation product is transformed into E. coli (e.g. DH5alpha strain) and produced there in an amount sufficient for the further transformation steps and purified. Due to the way of construction, it is also possible to obtain plasmids which contain the nucleic acid molecule in inverse orientation; in principle, however, these structures have the same functionality. E. coli clones containing the desired plasmid can be identified by PCR using the PCR primers mentioned, PCR4f and PCR4r; the resulting PCR product is 5.5 kb in length. These PCR analyses may be carried out using the Expand High Fidelity PCR system from Roche Applied Science, according to the specifications for the PCR reaction which are also supplied. In principle, it is possible to finally verify the cloning product by sequencing and sequence comparison with the DNA sequence depicted in Figure 6. A plasmid carrying the BamHI/EcoRV fragment is referred to as plasmid 2 and used below.

Example 5: Construction of plasmid 3 comprising the A. chrysogenum genes pcbAB and pcbC

Plasmid 1 of Example 1 is subsequently extended by the two cephalosporin biosynthesis genes pcbAB and pcbC to give plasmid 3. In nature, the genes pcbAB and pcbC are present in a genetically closely coupled form as biosynthesis gene cluster I and are described in S. Gutierrez, J Bacteriol. 173 (1991), pp. 2354-2365. Both genes are located in close proximity on an approx. 16 kb SnaBI/BfrI fragment and are provided with the aid of suitable lambda clones. Such lambda clones are isolated from a lambda gene bank containing DNA inserts of Acremonium chrysogenum ATCC48272. The construction of lambda gene banks is mentioned, for example, in T. Maniatis et al. (see above), as is the screening of lambda gene

banks by means of lambda-plaque hybridization. The DNA sequence information of the biosynthesis genes pcbAB and pcbC, required for this screening, can be made available, for example, by means of database requests (e.g. GENBANK). Examples of searh terms suitable for this database request are the names of the two biosynthesis genes (pcbAB, pcbC). Cloning of the two biosynthesis genes starts out from a lambda clone which is identified by means of plaque hybridization using sequence information of the pcbAB and pcbC genes, the said clone containing a DNA insert of at least 16 kb of DNA sequence. A SnaBI/BfrI restriction fragment of about 16 kb which comprises the two complete biosynthesis genes pcbAB and pcbC is essential for subsequent cloning. This fragment can be identified by PCR using the primers PCR5f and PCR5r which are defined by the following sequences:

Primer PCR5f	5'- AGG AGA GGC CGA AGA CGT CCC AGT A	(SEQ ID NO 16)
Primer PCR5r	5'- TTT CGC TTA GGG CTC GGA CGC T	(SEQ ID NO 17)

Plasmid 1 of Example 1 is extended by cloning the SnaBI/BfrI restriction fragment of approx. 16 kb according to SEQ ID NO 5, containing the two biosynthesis genes pcbAB and pcbC, into plasmid 1. The unique SmaI cleavage site on plasmid 1 is utilized for cloning. The two protruding ends of the approx. 16 kb SnaBI/BfrI restriction fragment are adapted with Klenow enzyme and ligated with the Sma1-cleaved plasmid 1.

The ligation product is transformed into E. coli (e.g. DH5alpha strain) and produced there in an amount sufficient for the further transformation steps and purified. Due to the way of construction, it is also possible to obtain plasmids which contain the nucleic acid molecule in inverse orientation; in principle, however, these structures have the same functionality.
E. coli clones containing the desired plasmid can be identified by PCR using the PCR primers mentioned, PCR5f and PCR5r; the resulting PCR product is 10.5 kb in length. These PCR analyses may be carried out using the Expand High Fidelity PCR system from Roche Applied Science, according to the specifications for the PCR reaction which are also supplied. In principle, it is possible to finally verify the cloning product by sequencing and sequence comparison with the DNA sequence depicted in Figure 5. A plasmid carrying the SnaBI/BfrI fragment is referred to as plasmid 3 and used below.

Example 6: Construction of plasmid 4 comprising the A. chrysogenum genes cefEF and cefG

Plasmid 1 of Example 1 is subsequently extended by the two cephalosporin biosynthesis genes cefEF and cefG to give plasmid 4. In nature, the genes cefEF and cefG are present in a genetically closely coupled form as biosynthesis gene cluster II and are described in S. Gutierrez, J Bacteriol. 174 (1992), pp. 3056-3064. Both genes are located in close proximity on an approx. 4.6 kb XbaI/SgrAI fragment and are provided with the aid of suitable lambda clones. Such lambda clones are isolated from a lambda gene bank containing DNA inserts of Acremonium chrysogenum ATCC48272. The construction of lambda gene banks is mentioned, for example, in T. Maniatis et al. (see above), as is the screening of lambda gene banks by means of lambda-plaque hybridization. The DNA sequence information of the biosynthesis genes cefEF and cefG, required for this screening, can be made available, for example, by means of database requests (e.g. GENBANK). Examples of search terms suitable for this database request are the names of the two biosynthesis genes (cefEF, cefG). Cloning of the two biosynthesis genes starts out from a lambda clone which is identified by means of plaque hybridization using sequence information of the cefEF and cefG genes, the said clone containing a DNA insert of at least 4.6 kb of DNA sequence. A XbaI/SgrAI restriction fragment of about 4.6 kb which comprises the two complete biosynthesis genes cefEF and cefG is essential for subsequent cloning. This fragment can be identified by PCR using the primers PCR6f and PCR6r which are defined by the following sequences:

Primer PCR6f	5'- TCG GGA GGT GGA GGA ATT CT	(SEQ ID NO 18)
Primer PCR6r	5'- ATC TTG CGC GCT GTT TCG AG	(SEQ ID NO 19)

Plasmid 1 of Example 1 is extended by cloning the XbaI/SgrAI restriction fragment of approx. 4.6 kb containing the two biosynthesis genes cefEF and cefG, into plasmid 1. The unique SmaI cleavage site on plasmid 1 is utilized for cloning. The two protruding ends of the 4.6 kb XbaI/SnaBI restriction fragment are adapted with Klenow enzyme and ligated with the Sma1-cleaved plasmid 1.

The ligation product is transformed into E. coli (e.g. DH5alpha strain) and produced there in an amount sufficient for the further transformation steps and purified. Due to the way of construction, it is also possible to obtain plasmids which contain the nucleic acid molecule in inverse orientation; in principle, however, these structures have the same functionality. E. coli clones containing the desired plasmid can be identified by PCR using the PCR primers mentioned, PCR6f and PCR6r; the resulting PCR product is 2.5 kb in length. These PCR analyses may be carried out using the Expand High Fidelity PCR system from Roche Applied Science, according to the specifications for the PCR reaction which are also supplied. In principle, it is possible to finally verify the cloning product by sequencing and sequence comparison with the DNA sequence depicted in Figure 7. A plasmid carrying the XbaI/SgrAI fragment is referred to as plasmid 4 and used below.

Example 7: Transformation of A. chrysogenum with plasmid 2

Transformation with plasmid 2 is illustrated below; however, it is also possible to use plasmid 3 and plasmid 4 accordingly and with comparable results (Examples 9 and 10).

Plasmid 2 is transformed into an A. chrysogenum strain (CEF-67605) by means of a standard procedure for protoplast transformation, it also being possible to use available A. chrysogenum strains such as ATCC48272 (see above) as alternatives. These methods are described, for example, in Nowak and Kück (see above) and involve selection of transformants on benomyl-containing nutrient agar. Nowak and Kück (see above) describe the properties of the modified tubulin gene CA_Tubulin(Tyr) used for this selection on plasmid 1 and, respectively, pCN3 and also the benomyl-containing nutrient agar required for selection. Transformants from such experiments are assayed for the presence of the essential parts of plasmid 2 by means of PCR. The PCR primers below allow such assaying:

Primer PCR7f 5'- GGG GCG GAG CCT ATG GAA AA (SEQ ID NO 20)

Primer PCR7r 5'- TCC AGC TCA CCT TGC TCC AG (SEQ ID NO 21)

The resulting PCR product is 9 001 bp in length. The PCR analyses may be carried out, for example, by means of the Expand Long Template PCR system from Roche Applied Science, according to the specifications for the PCR reaction which are also supplied.

Sensibly, a population of transformants with pCN3 is provided for control purposes in addition to a population of transformants with plasmid 2. These transformants can be analysed in PCR mixtures using the following PCR primers:

Primer PCR3f 5'- TTC CAT CCA GCA CCT CAC (SEQ ID NO 12)

Primer PCR3r 5'- CTT AAT GCG CCG CTA CAG (SEQ ID NO 13)

The resulting PCR product is 2 290 bp in length.

Example 8: Production and isolation of cephalosporin C from a transformed strain according to Example 7

The transformants generated in Example 7 are assayed as described in Example 3 and cephalosporin C is purified accordingly.

Among 550 transformants analysed, 7 strains (e.g. strain CET-98118) are found, whose cephalosporin C titre has increased by up to 10% in comparison with the non-transformed starting strain. Such strains may be used for production purposes on an industrial scale.

Example 9: Transformation of A. chrysogenum with plasmid 3 and subsequent production and isolation of cephalosporin C

Similarly to Example 7, an A. chrysogenum strain (CEF-67605) is transformed with plasmid 3, it also being possible to use available A. chrysogenum strains such as ATCC48272 as alternatives.

The transformants generated in this way are assayed as described in Example 3 and cephalosporin C is purified accordingly. Strains which have increased cephalosporin C titres in comparison with the non-transformed starting strain may be used for production purposes on an industrial scale.

Example 10: Transformation of A. chrysogenum with plasmid 4 and subsequent production and isolation of cephalosporin C

Similarly to Example 7, an A. chrysogenum strain (CEF-67605) is transformed with plasmid 4, it also being possible to use available A. chrysogenum strains such as ATCC48272 (see above) as alternatives.

The transformants generated in this way are assayed as described in Example 3 and cephalosporin C is purified accordingly. Strains which have increased cephalosporin C titres in comparison with the non-transformed starting strain may be used for production purposes on an industrial scale.